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The inhibitory effect of ddC on human immunodeficiency virus replication diminishes in cells that are chronically exposed to the drug

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Summary

One possible explanation for the failure of human immunodeficiency virus type 1 (HIV-1) antiretroviral inhibitors to block the clinical progression of the infection may be a failure to maintain adequate drug levels at the site of viral replication. We have previously found that exposure of human monoblastoid cells (U937) for several months to a therapeutically relevant concentration (0.1 μM) of 2′,3′-dideoxycytidine (zalcitabine, ddC) allowed the isolation of a drug-resistant cell line characterized by a normal drug transport but a reduced ability to accumulate 2′,3′-dideoxycytidine 5′-triphosphate (the active antiretroviral form of the drug). In this paper we show that the drug-resistant cells were indistinguishable from normal cells in terms of surface CD4 receptors. The susceptibility of parental and ddC-resistant U937 cells to infection by HIV-1 was similar, as measured by proviral DNA formation. However, HIV-1 p24 production and the number of infectious virus particles produced were significantly lower in the drug-resistant compared to control cells. Addition of 0.1 μM ddC inhibited viral production by up to 92% in the control cells but had no effect on ddC-resistant cells. Thus, human cells exposed to therapeutically relevant ddC concentrations for several months show a reduced ddC anabolism and allow ddC-sensitive HIV-1 to replicate in the presence of inhibitory ddC concentrations.

Keywords: Nucleoside analogues; cellular resistance; HIV; ddC.

Introduction

Treatment of HIV-1-infected patients with antiretroviral nucleoside analogues (AZT, zidovudine; ddI, didanosine; ddC, zalcitabine) most likely prolongs and improves the quality of life. However, existing therapies fail to block completely the clinical progression of the disease (Johnson & Hoth, 1993; Hirsch & D'Aquila, 1993).

The nucleoside analogues approved for use in HIV-1 disease are all reverse transcriptase (RT) inhibitors. Although these drugs do not decrease HIV-1 production from infected cells, they should protect new cells from becoming infected. There are two possible phenomena likely to contribute to the failure of RT inhibitors to provide clinical benefits. One explanation is the emergence of drug-resistant HIV-1 strains. The molecular bases for this phenomenon have been thoroughly investigated and are associated with mutations in HIV-1 RT loci (Larder *et al.*, 1989; Erice & Balfour, 1994). A second reason why patients progress toward the disease while on antiretrovirals could be a failure to maintain adequate drug levels in all potential sites of virus replication over long periods of time (Johnson & Hoth, 1993); these drugs are usually continued throughout life. This phenomenon is less known and has been poorly investigated. Recently we reported that U937 human monoblastoid cells, when exposed to therapeutically relevant concentrations of ddC for long periods of time, became resistant to drug mitochondrial toxicity (Magnani *et al.*, 1995a). We suggested that this phenomenon was associated with a reduced ability to accumulate 2′,3′-dideoxycytidine 5′-triphosphate (ddCTP, the active form of ddC) (Magnani *et al.*, 1995a,b). In this paper we report the failure of ddC to protect these resistant cells against ddC-sensitive HIV-1 infectivity. Thus, long-term exposure of U937 cells to ddC abrogates the drug's ability to protect the cell from wild-type HIV-1 infectivity and provides evidence for an additional mechanism of drug resistance.

Materials and Experimental Procedures

Cells and virus

U937 and C8166 T lymphoma cells (C8166 cells) were grown in RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 2 mM L-gluta-

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mine and 1% antibiotics. Isolation of the ddC-resistant cell line (U937-R) was obtained after exposure of U937 cells to 0.1 μM ddC (Fluka, Switzerland) for at least 1 month, as reported previously (Magnani *et al.*, 1995a). HIV-1 (IIIB strain) (Popovic *et al.*, 1984) was obtained by coculturing CEM-SS cells pretreated with Polybrene (8 μg mL^{-1}; Sigma) for 12 h with chronically infected H9 cells (3:1 ratio). The viral preparation was clarified at 2000 r.p.m., filtered and stored at −80°C. Titration of viral stock was performed using C8166 cells as described in detail elsewhere (Dianzani *et al.*, 1988).

Antibodies

Mouse MAbs against CD4 (MT310, IgG1κ), CD54 (ICAM-1, 6.5B5, IgG1κ), CD18 (MHM23, IgG1κ), CD11a (MHM24, IgG1κ), CD11b (2LPM19c, IgG1κ), CD11c (KB90, IgG1κ) and the isotype control were obtained from Dako (Milan, Italy); fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-mouse IgG antibodies were obtained from Cappel. The immunochemical characteristics of MAb MM4.17 (IgG2a) directed towards the P-glycoprotein extracellular domain have been previously described (Cianfriglia *et al.*, 1994).

Detection of mitochondrial DNA

Total cellular DNA was extracted and purified as reported elsewhere (Chen & Cheng, 1989). Mitochondrial DNA was linearized with *Bam*HI, analysed on a 0.8% agarose gel, transferred to nylon membranes and detected with a ^{32}P-labelled 1544 bp mitochondrial probe spanning nucleotide 2578 to 4122 in complete mitochondrial DNA (Anderson *et al.*, 1981).

Immunofluorescence analysis

Cells (6×10^5) were preincubated with 10 μL of FCS for 30 min at 4°C, then cells were washed twice with 2% FCS in PBS and incubated with an appropriate dilution of MAb for 30 min at 4°C. After two washes, cells were incubated with 40 μL of FITC-conjugated $F(ab')_2$ goat anti-mouse MAb diluted 1:50. After staining, cells were analysed on a bench-top flow cytometer (FACScan, Becton Dickinson) equipped with a 15 mW argon ion laser emitting light at a fixed wavelength of 488 nm. Fluorescence signals were collected in logarithmic mode (4-decade logarithmic amplifier) and in relative cell numbers per channel in a linear mode.

HIV production in U937 and U937-R cells

U937 and U937-R (1×10^6 cells) were centrifuged and resuspended in 1 mL of virus stock ($HIV\text{-}1_{IIIB}$) corresponding to 1 $TCID_{50}$ $cell^{-1}$. After 1 h of incubation at 37°C in a 5% CO_2 atmosphere, the cells were washed twice with PBS to remove unadsorbed virus particles and resuspended at a concentration of 2×10^5 cells mL^{-1} in RPMI-1640 medium containing 10% FCS. Cells were passaged in fresh medium twice a week. Supernatants were collected every 3–4 days and frozen for viral detection. HIV-1 production was assessed at established time points by measurement of HIV p24 antigen in the supernatant of infected cultures by a specific ELISA (NEK060, Du Pont). The amount of infectious virus in the culture supernatant at 9 days post-infection was analysed using C8166 T lymphoma cells (Dianzani *et al.*, 1988). The $TCID_{50}$ was calculated using the method of Reed & Muench (1938).

Antiviral assays

To evaluate the efficacy of ddC in inhibiting HIV-1 infectivity, both cell lines were infected with HIV-1 as reported above and cultured for 9 days in the presence of various concentrations of ddC. At the indicated times, aliquots of supernatants were collected and stored at −20°C for p24 determination or at −80°C for virus titration. Culture medium and ddC were changed 1 day before collection.

PCR analysis of HIV-1 proviral DNA

Total cellular DNA was isolated from the cells by lysis with 8 M urea, 0.3 M NaCl, 10 mM Tris–HCl pH 7.5 for 60 min at 37°C. Extraction was performed with phenol–chloroform–isoamyl alcohol (25:24:1) and then with chloroform–isoamyl alcohol (24:1). The DNAs were precipitated with ethanol and stored at −20°C until use in the PCR reaction. PCR analysis of HIV-1 proviral DNA was carried out using a commercial Kit (Perkin-Elmer Cetus). Genomic DNA (500 ng) corresponding to about 2.5×10^5 cells was used for each amplification in the presence of 200 μM each of dNTP, HIV-1 primers SK38 and SK39 (Ou *et al.*, 1988) specific for a highly conserved fragment of the *gag* open reading frame (ORF) at a final concentration of 0.375 μM, 0.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) in a 100 μL mixture containing 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$ and 0.01% gelatin.

The PCR process was achieved using the Perkin-Elmer Cetus DNA Thermal Cycler for 30 cycles of denaturation for 1 min at 95°C, annealing and extension for 1 min at 60°C followed by a final extension at 60°C for 10 min. The 115 bp PCR product was detected by the oligomer hybridization procedure whereby a ^{32}P-labelled SK19 oligonucleotide probe (from an internally conserved *gag* sequence spanning 1595 to 1635 bp) (Ou *et al.*, 1988; Kellog & Kwok, 1990) with [γ-^{32}P]ATP hybridizes in solution to one strand of the amplified product. The probe–target duplex was separated from the unhybridized probe by gel electrophoresis on a 10% polyacrylamide gel and was then autoradiographed (Kellog & Kwok, 1990).

Biochemical determinations

For the superoxide anion release assay, 3×10^5 cells were

PMA (0.5 μM)- and latex-stimulated and immediately processed for O^{2-} release as reported in Richard & Johnston (1984). For oxygen consumption, cells were suspended at 1×10^7 mL^{-1} in respiration buffer (0.25 M sucrose, 10 mM HEPES, 5 mM KH_2PO_4, 1 mM $MgCl_2$, 2 mM EGTA at pH 7.4) and transferred into the polarographic cell of an oxygraph (model 5300, Yellow Springs Instruments, Yellow Springs, Ohio, USA) equipped with a Clark-type electrode at 37°C under constant stirring. Basal respiration was measured in the presence of 5 mM glucose. Finally, a GSH assay was performed as reported in Beutler (1984).

Results

Expression of cell surface antigens

Long-term exposure of human cells (U937, CEM) to ddC results in time- and dose-dependent toxicity due to a continuous depletion of mitochondrial DNA (Fig. 1; Lewis & Dalakas, 1995).

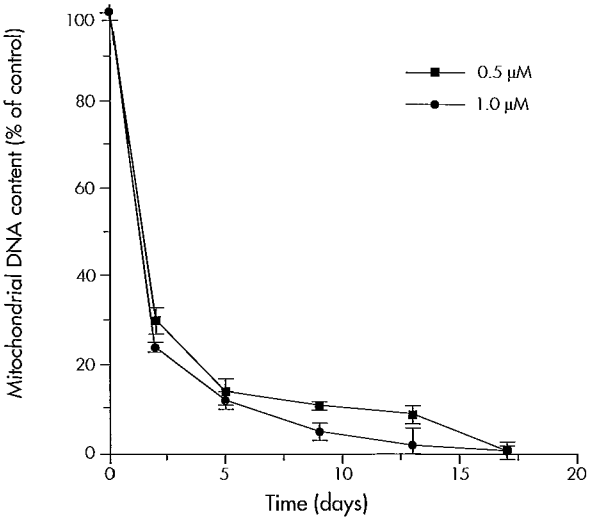
We investigated whether ddC resistance could be associated with increased expression of P-glycoprotein in U937-R cells. In fact, this protein, which is encoded by the MDR1 gene, is usually associated with the multidrug-resistant (MDR) phenotype of *in vitro*-selected drug-resistant cells (Edicott & Ling, 1989). However, neither U937 nor U937-R cells expressed MDR1-P-glycoprotein, as determined by reactivity of MAb MM4.17, directed towards the extracellular P-glycoprotein domain (data not shown).

Several others surface markers (CD4, CD11a, CD11b, CD11c, CD18, ICAM-1) were analysed in U937 and in U937-R cells. As shown in Fig. 2, U937-R cells exhibited a significantly increased expression of ICAM-1 and CD18 molecules. In contrast, surface CD4 expression was similar in both cell lines. The significance of the above increase is presently unknown. Similar CD4 expression suggests that both cell lines could be equally susceptible to HIV infection.

HIV replication in U937 and U937-R cells

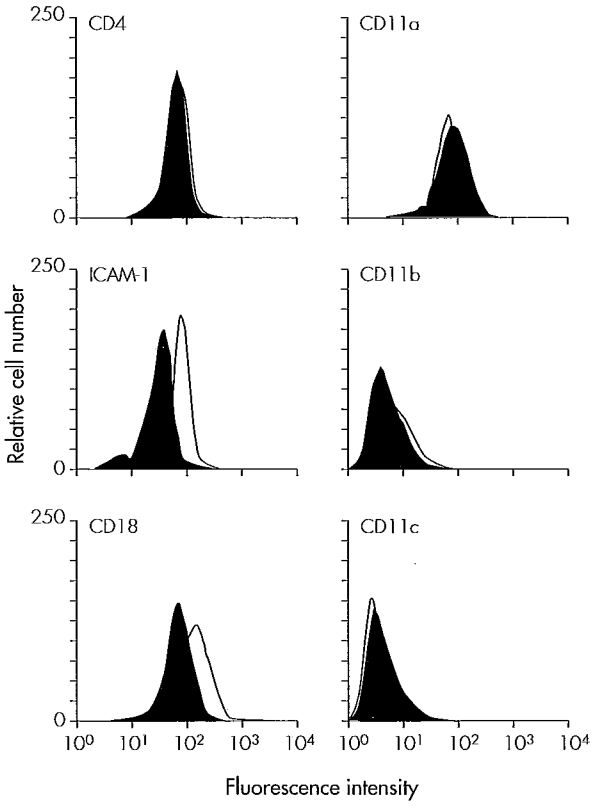
As shown in Fig. 3, the infection of parental U937 cells

Figure 1. Effect of ddC on mitochondrial DNA content.



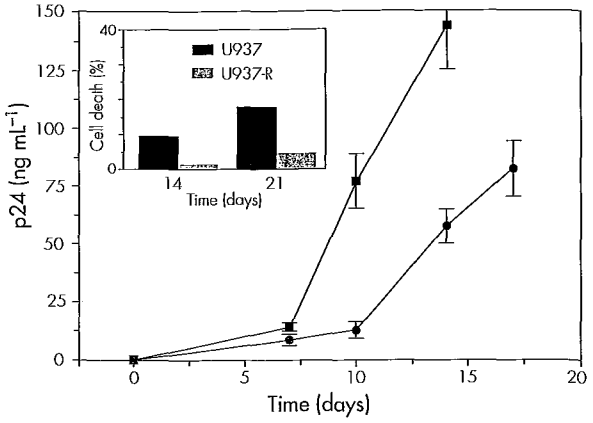
Total cellular DNA was extracted from U937 cells exposed to 0.5 or 1.0 μM ddC for different periods. Samples of DNA (0.6 μg, corresponding to about 3×10^5 cells) for each sample were digested with the restriction enzyme *Bam*HI, analysed on 0.8% agarose gel and transferred onto nylon membrane. Detection was with a ^{32}P-labelled 1544 bp human mitochondrial DNA probe. Values represent means ±SD of relative reduction of mitochondrial DNA in ddC-treated cells compared to control cells determined by laser scan densitometry of the autoradiograph with an LKB Ultrascan XL laser scan densitometer.

Figure 2. Expression of CD4, ICAM-1, CD18, CD11a, CD11b and CD11c in U937 and U937-R cells.



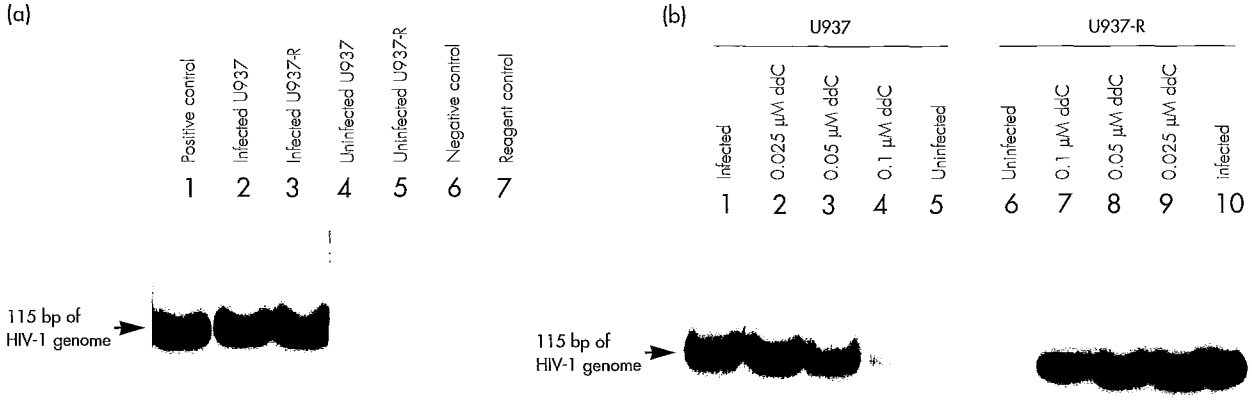
Cell surface molecule expression was determined by indirect immunofluorescence and FACScan analysis was conducted as detailed in Materials and Experimental Procedures. U937 cells, shaded; U937-R cells, outlined.

Figure 3. Kinetics of HIV-1 replication in U937 and U937-R cells.



Cells (10^6) were exposed to a m.o.i. of 1 for 1 h. Cells were then extensively washed with PBS to remove unadsorbed virus and cultured at 2×10^5 cells mL^{-1} in complete medium at 37°C in a 5% CO_2 atmosphere. Every 3 or 4 days cells were subcultured and samples of supernatant were assayed for HIV p24 antigen. Inset: cells were counted in duplicate with the trypan blue exclusion method to evaluate cell death. Values are the mean ±SEM calculated from three separate experiments. Differences in p24 production between the two cell lines are statistically significant ($P<0.01$).

Figure 4. PCR analyses of HIV-1 proviral DNA in U937 and U937-R cells.



One week after infection, cells were harvested, washed twice in cold 0.9% NaCl solution and processed as described in Materials and Experimental Procedures. A 115 bp section in the *gag* region of the HIV-1 genome was amplified by primers SK38/39 from 37500 cells, corresponding to about 75 ng of total cellular DNA. (a) Uninfected U937 and U937-R (lane 4 and 5) and infected U937 and U937-R (lane 2 and 3). (b) Infected U937 and U937-R cells (lane 1 and 10 respectively); U937 cells treated with 0.025, 0.05 and 0.1 µM ddC, respectively (lanes 2–4, percentage inhibition was 0, 27.3% and 86%, respectively) and U937-R cells treated with 0.1, 0.05 and 0.025 µM ddC, respectively (lanes 7–9, percentage inhibition was 20%, 3.2% and 0%). The percentage inhibition of proviral DNA in ddC-treated cells compared to infected cells was determined by laser scan densitometry as reported in Fig. 1. Positive control was plasmid DNA containing the genome of the HIVZ6 isolate (Perkin-Elmer Cetus) amplified from 375 copies (a, lane 1). Negative control was DNA from human placenta (7.5 ng) (a, lane 6). Detection was with a ^{32}P-end-labelled SK19 probe and autoradiography was carried out according to the instructions of the manufacturer (Perkin-Elmer Gene Amplimer HIV-1 Reagents).

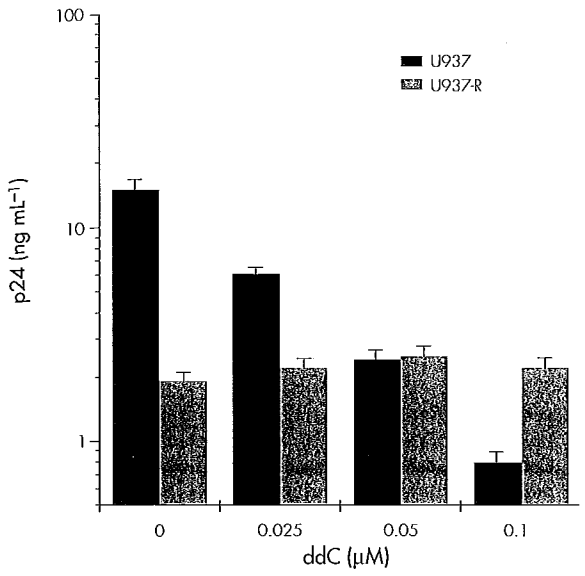
resulted in very high production of HIV-1 as evaluated by p24 protein release in the culture supernatant. In contrast, relatively low production of HIV-1 was observed in U937-R cells. Syncytia and cell death were observed in HIV-infected culture starting on day 7. Both were more pronounced and seen earlier in U937 cells (Fig. 3).

One week after infection the amount of proviral DNA was also analysed by a semi-quantitative PCR assay (Fig. 4a). Both cell lines contained approximately the same amount of proviral DNA, determined by laser scan densitometry of the autoradiograph bands.

ddC inhibition of HIV-1 infectivity

We examined the antiviral activity of several doses of ddC (0.025, 0.05 and 0.1 µM) in U937 and U937-R cells exposed to HIV-1. As shown in Fig. 5, ddC exerted a potent inhibitory effect on HIV-1 production in U937 cells (0.016 µM ddC induced 50% suppression of p24

Figure 5. The effect of ddC on HIV-1 replication in U937 and U937-R cells.



Both cell lines were infected and cultured in the presence or absence of different concentrations of ddC. Medium with or without the drug was completely replaced every 3 days, and 24 h before collecting culture supernatant for viral detection. At day 9, HIV-1 replication was evaluated by measuring p24 antigen in the supernatant. (The ninth day was selected because at this time there was a considerable production of p24 antigen but the toxic effect of the drug was not yet evident; similar results were also obtained on day 10 and 12.) Values are the mean ±SEM calculated from three separate experiments.

release in culture supernatant). No decrease in p24 production was observed in HIV-infected U937-R cells treated with ddC. Similar results were obtained when infectious virus was measured in the supernatant of cells treated with the drug. Again, 0.1 μM ddC induced a 3–4-fold decrease in infectious virus production in U937 cells (basal production: about 237 $TCID_{50}$ mL^{-1}). ddC did not significantly affect release of infectious virus in U937-R culture supernatants (basal: 75 $TCID_{50}$ mL^{-1})

These results were also confirmed by PCR detection of proviral DNA in both cell lines in the presence of increasing ddC concentrations (Fig. 4b). No toxicity (as assessed by trypan blue exclusion) was observed after 9 days in U937 cells treated with 0.1 μM ddC.

Some biochemical properties of U937 and U937-R cells

In an attempt to investigate the reason(s) for reduced virus production in HIV-1-infected U937-R cells compared to controls, we determined some relevant biochemical properties. U937-R cells showed an increased oxygen consumption (21.6±1.6 versus 16±0.9 nmol min^{-1} 10^7 $cells^{-1}$ in controls), an increased superoxide anion release (7±1.0 versus 4.3±0.8 nmol min^{-1} 10^7 $cells^{-1}$ in controls) and also contained a higher concentration of reduced glutathione (38±2.5 versus 25±1.2 nmol mg^{-1} protein in U937 cells).

Discussion

The results of this study suggest that in addition to viral resistance to antiviral drugs (Johnson & Hoth, 1993; Hirsch & D'Aquila, 1993; Erice & Balfour, 1994), cellular resistance can contribute to the failure of antiviral drugs in HIV-1-infected patients. ddC, as well as the other nucleoside analogues licensed for HIV-1 therapy, must first be phosphorylated by specific cellular kinases in order to exert their pharmacological activity (Broder, 1990). Unfortunately, the phosphorylated nucleoside analogues also exert cytotoxic effects (Chen & Cheng, 1989, 1992). It must be considered that these drugs are administered for long periods of time. As a consequence, it is to be expected that cells develop drug resistance mechanisms. One possible mechanism of drug resistance, which has already been documented in cells exposed to antitumour nucleoside analogues, concerns the inactivation of the enzyme responsible for drug phosphorylation, namely deoxycytidine kinase (Ruiz van Harperen & Peters, 1994). This was not the case in the drug-resistant cells described in this paper, although deoxycytidine kinase had a reduced affinity and reduced V_{max} for ddC (Magnani *et al.*, 1995a). ddC-sensitive and ddC-resistant cells were indistinguishable by flow cytometry in term of surface CD4 receptors. Furthermore, PCR analysis of proviral DNA content showed that HIV-1 infects both cell lines equally well. However, the drug-resistant cells produced less p24 and less HIV-1 infectious particles than control cells. We have previously shown that ddC-resistant cells also contain an increased number of mitochondria per cell (Magnani *et al.*, 1995b). These organelles, the sites of oxygen metabolism, produce significant amounts of reactive oxygen species (ROS). ROS have been suggested to activate HIV-1 expression through activation of NF-κB (Schreck *et al.*, 1991; Muller, 1992), but this does not appear to be the case in our system where HIV-1 production is impaired. Furthermore, a number of studies (Aukrust *et al.*, 1995; Droge *et al.*, 1994; Staal *et al.*, 1992; Pace & Leaf, 1995) have reported an altered intracellular redox balance in HIV-1-infected patients and that *in vitro*, glutathione, glutathione esters and N-acetyl-cysteine strongly affect viral production by modifying the intracellular glutathione redox state (Kalebic *et al.*, 1991; Roeder *et al.*, 1990; de Quary *et al.*, 1992). The determination of reduced glutathione in our U937-R cells provided clear evidence for

an increased GSH concentration. Thus, at least in part, the reduced viral production observed in these cells may be contributed to by an increased glutathione concentration. It is worth noting that U937-R cells also contain a reduced amount of 2′-dCTP (3.73±0.22 pmol 10^6 $cells^{-1}$) compared to U937 (6.57±0.32 pmol 10^6 $cells^{-1}$) (Magnani *et al.*, 1995b). Since recent reports (Gao *et al.*, 1994; Meyerhas *et al.*, 1994) have shown that the level of cellular dNTPs can also strongly affect HIV-1 infectivity and replication, it is difficult to evaluate what percentage of the reduced viral production observed in the U937-R line was due to an increased GSH concentration and/or to a reduced 2′-dCTP concentration. Thus, the molecular bases for the inhibition of HIV-1 expression in ddC-resistant cells need further investigation.

The most relevant information derived from the studies reported in this paper concerns the absence of antiviral activity of ddC in cells exposed for 2 months to therapeutically relevant ddC concentrations (0.1 μM). This *in vitro* situation may represent the *in vivo* situation in which uninfected cells in HIV-1 patients are continuously exposed to antiviral nucleoside analogues. It can be speculated that such prolonged exposure prevents the cells from being protected from HIV-1 infection once this occurs. This phenomenon could be responsible for the long-term failure of nucleoside analogues in preventing the progression of the disease and the spreading of the virus, and may be responsible for failure of an early antiviral therapy in HIV-1-infected patients (see the Concorde study). It is interesting to note that a similar mechanism was also documented in AZT-resistant cell lines and in AZT-treated patients (Avramis *et al.*, 1993; Wu *et al.*, 1995; Dianzani *et al.*, 1994). Furthermore, Cinatl *et al.* (1993) showed that MOLT-4 cells grown for several months in the presence of 0.5 μM ddC and subsequently in the absence of the drug for 1 month, were much less sensitive to ddC inhibition of HIV-1 production. Again, long-term exposure of cells to nucleoside analogues appears to abrogate the antiviral effect of the drug.

In conclusion, drug-induced cell resistance may severely hamper the antiviral properties of nucleoside analogues. An appropriate administration schedule, considering alternating treatment regimes with drugs that are activated by different cellular enzymes, is worthy of further evaluation in patients expected to receive these drugs for many years.

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